

NEUROTOXIC DESTRUCTION OF THE SEROTONINERGIC  
INNERVATION OF THE RAT SUBCOMMISSURAL ORGAN IS  
FOLLOWED BY REINNERVATION THROUGH COLLATERAL  
SPROUTING OF NON-MONOAMINERGIC NEURONS

by

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Running title: Non-monoaminergic reinnervation of the denervated SCO

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### Summary

Specific neurotoxic destruction of the serotonergic innervation of the subcommissural organ of the rat is followed by an efficient reinnervation by collateral sprouting of non-monoaminergic axons, which normally do not innervate the SCO cells. Morphologically, the reinnervating fibers totally replace the serotonergic synapses lost by the lesion, but, functionally, they fail to substitute for the potent inhibitory control of secretory activity normally exerted by the serotonergic innervation. It is possible that the observed reinnervation by foreign synapses explains why the regrowing serotonergic neurons fail to reestablish their connections with the SCO.





## Introduction

It has been observed that after axotomy induced by specific neurotoxic drugs, central monoaminergic neurons have the capacity of regenerative growth, and are able to reestablish terminal innervation patterns (e.g. Nobin et al., 1973; Nygren and Olson, 1977; Wiklund et al., 1978). An important aspect of this regeneration of monoaminergic connections is the absence of glial scar formation, which has been proposed to be a limiting factor in central nervous regeneration after mechanical axotomy (cf. Windle, 1955; Clemente, 1964). The regrowth of chemically lesioned axons is favoured by a virtually unaltered tissue milieu, where postulated mechanisms of guidance and recognition presumably are intact. Several aspects of the regeneration of monoaminergic systems remain, however, to be clarified. For non-monoaminergic systems it has been well established by experiments in for example the septum (Raisman, 1969; Raisman and Field, 1973), hippocampus (for review, Cotman and Lynch, 1976), visual system (Lund and Lund, 1971; Goodman et al., 1973) and red nucleus (Nakamura et al., 1974; Tsukahara et al., 1975; Murakami et al., 1976) that destruction of one afferent system will often lead to plastic remodelling of the neuropil, which involves the production of new synapses by intact systems to replace the synapses lost by the lesion. This phenomenon is generally known as reinnervation through collateral sprouting. Until now, it has not been known, however, if destruction of monoaminergic afferents to an area of C.N.S. will elicit collateral sprouting in remaining afferents. In case this type of remodelling occurs, it will be of fundamental interest to determine if the regrowing monoaminergic nerve fibers in their reinnervation process replace the foreign terminals.





In a preceeding paper (Møllgård and Wiklund, 1978) we have reported that the subcommissural organ (SCO) of the rat is heavily innervated by serotoninergetic nerve fibers, which terminate with abundant asymmetric synaptic contacts on the perikarya and basal processes of ependymal and hypendymal cells. The effect of intraventricular administration of the specific neurotoxic drugs 5,6- and 5,7-dihydroxytryptamine (5,6-DHT and 5,7-DHT) on the innervation was investigated with fluorescence histochemistry and electron microscopy. Both drugs were found to cause a virtually complete destruction of all serotoninergetic terminals in the SCO. Further, it was observed fluorescence histochemically that the serotoninergetic neurons fail to reinnervate the SCO. In the present report we demonstrate a reinnervation of the SCO by non-monoaminergic nerve fibers. As far as we are aware, this constitutes the first clear example of reinnervation by collateral sprouting elicited by the removal of a monoaminergic afferent system.

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## Material and Methods

This study includes some of the experimental material which was described in the accompanying paper (Møllgård and Wiklund, 1979).

### Administration of neurotoxic drugs.

Female Sprague-Dawley rats (180-220 g body weight) under barbiturate anaesthesia (Brietal<sup>R</sup>, Lilly 40 mg/kg) were injected stereotaxically in the anterior lateral ventricle with 50 or 75 µg 5,6-DHT or 150 µg 5,7-DHT (both drugs calculated as free base). The neurotoxic drugs were administered in a volume of 20 µl saline containing 0.2 mg/ml L-ascorbic acid. Normal rats and rats injected intraventricularly with 20 µl saline served as a control. (For a discussion of these drugs and their neurotoxic properties, cf. the preceeding paper, Møllgård and Wiklund, 1978, and the review by Baumgarten et al., 1977).

### Electron microscopy.

Survival times for experimental animals included 1 month (4 rats), 3 months (2 rats) and 8 months (4 rats). Two of the experimental animals which survived for 8 months after the first injection received a second injection of 5,6-DHT (75 µg) two days before sacrifice. Control and experimental rats were fixed by transcardiac perfusion of a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Following 15-20 min perfusion the brains were removed and placed in fresh fixative for 6 h. Tissue blocks containing the SCO were then postfixed for 2 h in 2% OsO<sub>4</sub> in the same buffer, block-stained with uranyl acetate for 1 h, dehydrated in increasing concentrations of ethanol and embedded in Epon. One micrometer thick sections were stained with toluidine blue. For electron microscopy of the SCO silver-to-grey thin sections were cut from selected areas and stained with uranyl acetate and lead citrate.





## Results

Most of the material investigated as part of the present study was obtained from rats, which had survived for one month after administration of the neurotoxic drug. The SCO of animals surviving for 3 or 8 months was also investigated. Fluorescence histochemically, we found no evidence for a restoration of the serotonergic SCO innervation after these survival periods (see Møllgård and Wiklund, 1978).

Electron microscopically it was found that many cells contained amorphous electron dense elements, which were interpreted as phagocytosed debris from the degenerated serotonergic fibers originally innervating the SCO cells. Fragments of degenerated boutons were characteristically found in glial cells of the deep hypendyma (Fig. 1), but also SCO hypendymal and basal processes of SCO ependymal cells contained such remnants of the 5-HT fibers. Approximately the same amount of phagocytosed material was found after one month as after longer survival times (3 and 8 months) indicating that no, or very little, further degradation of the phagocytosed nerve endings occurs.

Investigation of the basal SCO revealed that a surprisingly dense reinnervation of the SCO cells had taken place. Great numbers of relatively large boutons of unmyelinated nerve fibers were seen to engage in typical synaptic contacts with cells of the SCO. The distribution of reinnervating synapses was similar to that of the original 5-HT innervation. Thus, synapses were located very close to the nuclei of SCO cells (Figs. 2a, b), but basal processes of ependymal and processes of hypendymal SCO cells were also contacted (Fig. 3). The same reinnervating





bouton was quite often seen to engage in synaptic contacts with more than one SCO cell (Fig. 2b). The synaptic junctions showed the characteristics of asymmetric Gray's type I synapses. The synaptic clefts and membrane specializations were of dimensions similar to those of the normally occurring 5-HT synapses. Large boutons with a morphology similar to that of the newly formed synaptic terminals were observed in very close contact to SCO cells without forming synaptic junctions (Fig. 4).

The ultrastructural features of the boutons reinnervating the SCO cells were analyzed in detail on a number of high power electron micrographs (X 60,000) from the SCO of 4 animals surviving for 1 month after treatment. The average dimensions of the reinnervating boutons were  $2 \times 1.5 \mu\text{m}$  (long and orthogonal diameters). This means that they are considerably larger than the 5-HT boutons they have replaced (cf. Møllgård and Wiklund, 1978). The reinnervating boutons often contained numerous vesicles and more mitochondria than the original 5-HT terminals (Fig. 5). The vesicle population of the reinnervating boutons was rather similar to that of the normal 5-HT boutons, and consisted of small, clear round vesicles with a mean diameter of 55 nm and elliptic vesicles averaging  $65 \times 45 \text{ nm}$  (for classification criteria see Møllgård and Wiklund, 1978). However, while the normally occurring 5-HT boutons contain a majority of elliptical vesicles, the reinnervating boutons were marked by a predominance of round vesicles. In addition to small clear vesicles, a few large dense core vesicles were usually observed in the reinnervating terminals (Fig. 5).

In an attempt to demonstrate electron microscopically that the newly formed synapses were not serotonergic, the initial





injection of serotonin neurotoxin was repeated several months later. Experimental animals sacrificed 2 days after the second injection exhibited intact synapses in the SCO which confirm the non-serotonergic nature of the fibers reinnervating the SCO.

A description of the synaptic input to somata and large dendrites of the SCO-associated neurons (Kimble and Møllgård, 1975) is not the purpose of the present communication, but it is worth mentioning that the ultrastructural features of this input are very similar to those of the reorganized synaptic input to the denervated SCO cells. The neurons are predominantly contacted by large varicosities - about 1.5  $\mu\text{m}$  in diameter - with many mitochondria and abundant small clear synaptic vesicles. More than 50% of the vesicles are of the round variety with a diameter of about 55 nm. Though many of these boutons engage in axo-somatic synapses with the SCO-associated neurons they show the characteristics of asymmetric Gray's type I synaptic junctions.



## Discussion

Central monoaminergic neurons have been found to demonstrate remarkable capacities for regenerative growth in experiments involving mechanical or electrolytic lesions (Björklund et al., 1971; Katzman et al., 1971), transplantation of artificial target tissue (Björklund and Stenevi, 1971; Svendgaard et al., 1975), and chemical lesioning with selective neurotoxic drugs (Baumgarten et al., 1973; Björklund et al., 1973; Nobin et al., 1973; Nygren et al., 1974; Wiklund et al., 1978; Björklund and Lindvall, 1979). The experiments with neurotoxic drugs are of particular interest, since in these studies the lesioned monoaminergic axons were able to reestablish terminal plexuses in denervated target areas. It can, however, be questioned if these examples of extensive regeneration of interrupted monoaminergic connections are representative for all C.N.S. tissue, or if the monoaminergic neurons in regenerative growth differ in some fundamental aspect from other classes of central neurons. Therefore it is of interest to correlate the properties of growth and plasticity of monoaminergic neurons to what is known from other unmyelinated and also from myelinated neuron systems.

Interruption of a non-monoaminergic afferent system in the C.N.S. may elicit a plastic remodelling of the neuropil, whereby intact systems extend to fill the vacated synaptic space. This phenomenon was first observed in the C.N.S. by Liu and Chambers (1958) who worked with dorsal root afferents in the spinal cord. Since Raisman's demonstration of collateral reinnervation at the ultrastructural level in the septum (Raisman, 1969; Raisman and Field, 1973) this concept has become accepted as a fundamental





aspect of C.N.S. response to injury. More recently Tsukuhara and associates have investigated the functional properties of synapses formed in the process of collateral sprouting reinnervation of the red nucleus (Tsukuhara et al., 1975; Murakami et al., 1976).

Relatively little is known about central monoaminergic systems in relation to plastic remodelling of neuropil through collateral reinnervation. A few studies have shown that catecholaminergic systems can respond by growth to the removal of non-monoaminergic afferents (Moore et al., 1971; Stenevi et al. 1972), and some recent data from experiments with transplantation of fetal brain tissue indicate that available synaptic space can influence the growth of immature central catecholaminergic as well as serotonergic neurons (Björklund et al., 1976). Previous investigations have, however, not shown if destruction of monoaminergic innervation elicits collateral sprouting of remaining afferents, and therefore it is not known, whether a plastic remodelling of the neuropil will influence the regeneration of monoaminergic connections.

It is most convenient to investigate collateral sprouting after removal of a monoaminergic input at the ultrastructural level in areas of C.N.S. where the monoaminergic innervation constitutes a substantial part of the afferent fibers to a well-defined target area. Furthermore, the sites of neuron-target cell interaction should be readily recognizable in the electron microscope. The serotonergic innervation of the rat SCO meets these criteria since the serotonergic boutons forming abundant asymmetric synapses on the characteristic cells of the SCO constitute the major, if not only, innervation of the organ (Møllgård and Wiklund, 1979).



Neurotoxic destruction of the serotonergic SCO innervation by intraventricular administration of 5,6- or 5,7-DHT was in the present study found to lead to an efficient reinnervation of the SCO cells by non-monoaminergic boutons. The reinnervating boutons were considerably larger, had a different synaptic vesicle content, and more mitochondria than the 5-HT boutons they replaced, but the synaptic junctions formed with the denervated SCO cells had the same ultrastructural appearance as the original 5-HT synapses. The reinnervating boutons did not, however, seem to substitute the serotonergic innervation functionally, since the marked elevation of secretory activity caused by interruption of the inhibitory 5-HT mediated control (Møllgård et al., 1978; Møllgård and Wiklund, 1979) remained in spite of the total reinnervation observed. Presumably, the inhibition of the secretory activity of the SCO is mediated by interaction with specific receptors sensitive for serotonin. The origin of the fibers replacing the lost 5-HT input has not been determined, but the boutons are very similar to afferents of the nearby SCO-associated neurons.

The efficient collateral reinnervation of the denervated SCO presents the regenerating 5-HT neurons with the problem of displacing the foreign boutons from the synaptic sites they have occupied in order to reconstitute normal conditions. The observed failure of the 5-HT neurons to reinnervate the SCO (Møllgård and Wiklund, 1979) suggests, however, that the regrowing 5-HT axons are unable to replace the non-monoaminergic innervation. They also seem unable to elicit formation of new synaptic junctions, which could exist parallel to the non-monoaminergic boutons. These observations could indicate that collateral reinnervation





when it occurs, could limit the successful regeneration of monoaminergic connections. Available data seem, indeed, to be compatible with such an interpretation. Ultrastructural investigations of 5-HT innervation in several areas of C.N.S. (for references, see the preceeding paper) have shown that 5-HT axons often terminate with the formation of very few synaptic junctions. Actually, the varicose 5-HT axons in these areas seem to end in the neuropil in a fashion reminiscent of the free terminal arborizations of the peripheral autonomic neurons. It is possible that regenerating 5-HT axons can reestablish innervation only in areas which receive "non-synaptic" monoaminergic innervation, while in areas of "synaptic" 5-HT innervation the proliferation of other afferents will lead to a permanent replacement of the 5-HT synapses. This interpretation receives some support from observations in the suprachiasmatic nucleus. The 5-HT terminals that innervate this hypothalamic nucleus seem to terminate with a formation of abundant synaptic contacts (Nojyo and Sano, 1978) and, after 5,6-DHT induced denervation, the regrowing 5-HT axons fail to reinnervate the suprachiasmatic nucleus (Björklund et al., 1973; Wiklund, unpublished results).

Alternative interpretations of the present data are possible. Both the SCO and the suprachiasmatic nucleus are located comparatively far from the proximal stumps of the axons lesioned by the neurotoxic drug. Therefore, the regrowing axons have a long distance to travel before reaching the denervated SCO and suprachiasmatic nucleus. Östberg et al. (1976) have shown that synaptogenesis can be limited by the number of synaptic connections the regenerating axons are able to establish, and



inferred that in the normal situation neurons may be close to their maximal extent. It is known that lateral ventricular injection of 5,6- or 5,7-DHT in addition to the axotomy causes degeneration of a certain number of 5-HT cell bodies (Björklund et al., 1973; Nygren et al., 1977). In the regenerative situation the reduced number of 5-HT neurons are therefore meeting the demand of reinnervating a larger terminal area than they originally covered. The situation is further complicated by the observation that the outgrowing 5-HT fibers favour proximal target areas of the brainstem, where abnormally dense hyperinnervations are formed in several nuclei (Nobin et al., 1973; Wiklund et al., 1978). For distant target areas, like the SCO, there may simply be insufficient numbers of 5-HT axons available for reinnervation. Or, the limited number of 5-HT axons reaching these distal regions may preferentially reinnervate surrounding "non-synaptic" target areas. Whether or not the regrowing 5-HT neurons are capable of forming new synapses should for these reasons preferably be investigated in more proximal target areas. Such investigations are now in progress.





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## Legends

Fig. 1. Rat SCO one month after intraventricular injection of 5,7-DHT. Phagocytosed material from degenerated 5-HT fibers is accumulated in a large glial cell process. The intermembranous compartment of the phagocytosed mitochondria exhibits the characteristic electron dense appearance.  
Bar: 0.5  $\mu$ m.

Figs. 2a & b. One month after administration of 75  $\mu$ g 5,6-DHT no 5-HT fibers can be detected in the SCO by fluorescence microscopy (see also Fig. 2 in the preceeding paper). Electronmicroscopically it is obvious that a dense reinnervation has taken place. In both micrographs the newly formed synapses are located close to the SCO cell nuclei (N). Note the ER cisternae (arrows) in 2a and the double synaptic contact in 2b.  
Bars: 0.5  $\mu$ m.

Fig. 3. A presynaptic terminal in synaptic contact with a basal SCO process. Note the increased extracellular space and the presence of slender processes, some of which partly envelope the terminal originating from SCO cells (arrow). However, the gap junctions between the three processes in the upper part of the micrograph (arrow-heads) are characteristic of astrocytes. Note also the characteristic ER



cisternae.

Bar: 0.5  $\mu$ m.

Fig. 4. A large bouton is in close contact with an SCO cell. The short distance from the point of contact to the SCO cell nucleus is similar to what is found in both normal and reinnervated rat SCO. Note the many mitochondrial profiles.  
Bar: 0.5  $\mu$ m.

Fig. 5. Three newly formed synaptic boutons have established contact with two SCO cell processes. Two boutons in the upper part of the micrograph form a punctum adherens-like contact (PA). Note the considerable size of the boutons and the numerous mitochondrial profiles. Arrows point to some slender processes which partially envelope the boutons.  
Bar: 0.5  $\mu$ m.























